

(19)



Europäisches Patentamt

European Patent Office

Office européen des brevets



(11)

**EP 0 370 989 B1**

(12)

**EUROPEAN PATENT SPECIFICATION**

(45) Date of publication and mention  
of the grant of the patent:  
**04.09.1996 Bulletin 1996/36**

(51) Int Cl.<sup>6</sup>: **C12N 15/12, C12P 21/02,  
C12N 5/16**

(21) Application number: **89870181.8**

(22) Date of filing: **20.11.1989**

(54) **Human vascular permeability factor**

Menschlicher Gefäßpermeabilitätsfaktor

Facteur de perméabilité vasculaire humain

(84) Designated Contracting States:  
**AT BE CH DE ES FR GB GR IT LI LU NL SE**

(30) Priority: **21.11.1988 US 274061**  
**10.07.1989 US 377037**

(43) Date of publication of application:  
**30.05.1990 Bulletin 1990/22**

(73) Proprietor: **GENENTECH, INC.**  
**South San Francisco California 94080 (US)**

(72) Inventors:

- **Keck, Pamela Jean**  
**St. Louis, MO 63146 (US)**
- **Feder, Joseph**  
**University City, MO 63130 (US)**
- **Connolly, Daniel Thomas**  
**Manchester, MO 63021 (US)**
- **Olander, Jitka Vera**  
**University City, MO 63130 (US)**

(74) Representative: **Armitage, Ian Michael et al**  
**MEWBURN ELLIS**  
**York House**  
**23 Kingsway**  
**London WC2B 6HP (GB)**

(56) References cited:  
**WO-A-84/02135**

- **JOURNAL OF BIOLOGICAL CHEMISTRY**, vol. 264, no. 33, 25 November 1989, Baltimore, MD (US); D.T. CONNOLLY et al., pp. 20017-20024
- **MONOKINES AND OTHER NON-LYMPHOCYTIC CYTOKINES**, Alan R. Liss Inc., 1988, New York, NY (US); G. BECK et al., pp. 325-328
- **CANCER RESEARCH**, vol. 46, November 1986, Philadelphia, PA (US); D.R. SENGHER et al., pp. 5629-5632
- **JOURNAL OF NEUROSURGERY**, vol. 69, August 1988, Baltimore, MD (US); G.R. CRISCUOLO et al., pp. 254-262

**370 989 B1**

## Description

### Background of the Invention

5 This invention relates to a novel human vascular permeability factor and to a cDNA clone representing the full size human vascular permeability factor protein.

Vascular permeability factors (VPFs) are proteins originally obtained from a variety of tumors which cause a rapid and reversible increase in blood vessel permeability when nanogram amounts are injected under the skin of a warm blooded mammal. VPF activity has been found in tumor ascites fluid from guinea pigs, hamsters and mice and is  
10 secreted by these tumors and a variety of tumor cell lines in vitro according to Senger et al., Science 219, 983-985 (1983).

In U.S. Patent 4,456,550, a purified VPF is described which has the following characteristics:

(a) in an aqueous solution (0.01 M  $\text{Na}_3\text{PO}_4$ , pH 7) whose concentration of NaCl is varied linearly, VPF is eluted  
15 from a heparin-Sepharose chromatography column in a peak centered at 0.4 NaCl;

(b) in an aqueous solution of  $\text{Na}_3\text{PO}_4$ , pH 7.0, whose concentration is varied linearly, VPF is eluted from a hydroxylapatite column in a peak centered at 0.25 M  $\text{Na}_3\text{PO}_4$ ; and

(c) when subjected to SDS gel electrophoresis in a polyacrylamide slab gel (0.375 M tris-HCl, pH 8.8, 0.1% SDS) at 35 milliamps and 4°C., VPF is localized to a region corresponding to a molecular weight between 34,000 and 45,000 daltons.  
20

VPF of the foregoing characteristics was thus purified about 1800 fold from serum-free conditioned medium of guinea pig tumor cell culture or 10,000 fold from ascites fluid by a series of steps consisting of:  
25

(a) affinity chromatography with a column of heparin-Sepharose;

(b) chromatography with a column of hydroxylapatite; and  
30

(c) sodium dodecylsulfate/polyacrylamide gel electrophoresis.

According to said patent, as little as 200 ng ( $5 \times 10^{-12}$  moles) of this purified VPF increased the vascular permeability equivalent to 1.25  $\mu\text{g}$  ( $4 \times 10^{-9}$  moles) of histamine. Histamine is a standard permeability mediator described by Miles and Miles, J. Physiol. 118, 228-257 (1952). The VPF is said to have therapeutic value insofar as it enables blood nutrients to reach tissue with increased need for nutrients, as in wound healing.  
35

According to Folkman and Klagsbrun, Science 235, 442-447 (1987), VPF causes leakage of proteins, including fibrinogen, from blood vessels, thereby initiating the formation of a fibrin gel which, in turn, may play a role in angiogenesis. See also Dvorak et al., J. Immunol. 122(1), 166-174 (1979); Dvorak, N. Engl. J. Med. 315, 1650-1659 (1986); Kadish et al., Tissue & Cell 11, 99 (1979); and Dvorak et al., J. Natl. Cancer Inst. 62, 1459-1472 (1979).  
40

In an application assigned to a common assignee, a method of stimulating endothelial cell growth is provided which comprises subjecting said cells to a growth stimulating amount of a highly purified VPF. The highly purified VPF derived from guinea pig tumor cells has the following characteristics:

(a) it has a  $M_r$  about 34,000 - 40,000 as determined by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS/PAGE);  
45

1 10  
A P M A E C C C C N

(a) <sup>17</sup>F M D V Y Q <sup>23</sup>R,

5 (b) <sup>33</sup>V D I F Q E Y P D E I E <sup>45</sup>Y, and

(c) <sup>46</sup>I F K P S C V P L M <sup>56</sup>R.

10 The cDNA sequence and corresponding predicted amino acid sequence of the hVPF is shown in the following 1195 bp sequence which includes portions of the 5'- and 3'- noncoding regions. Nucleotides are numbered in the lefthand column, beginning with No. 1. Amino acids are numbered from above. The predicted amino acid sequences from the cDNA clone for amino acid positions 1-18, 17-23, 33-45 and 46-56 exactly match amino acid sequences determined by amino acid sequencing of the N-terminus and of three different tryptic peptides. These sequences are  
15 underlined. The single N-glycosylation site at Asn-75 is boxed. The stop codon is designated with three asterisks.

20

25

30

35

40

45

1 GCGCAGACAG TGCTCCAGCG CGCGCGCTCC CCAGCCCTGC CCGGCCTCGG  
 51 GCCGGGAGGA AGAGTAGCTC GCCGAGGCGC CGAGGAGAGC GGGCCGCCCC  
 101 ACAGCCCGAG CCGGAGAGGG ACGCGAGCCG CGCGCCCCGG TCGGGCCTCC  
 -26 -20  
 151 Met Asn Phe Leu Leu Ser Trp Val His Trp Ser Leu  
 GAAACC ATG AAC TTT CTG CTG TCT TGG GTG CAT TGG AGC CTT  
 -10  
 193 Ala Leu Leu Leu Tyr Leu His His Ala Lys Trp Ser Gln Ala  
 GCC TTG CTG CTC TAC CTC CAC CAT GCC AAG TGG TCC CAG GCT  
 +1 10  
 235 Ala Pro Met Ala Glu Gly Gly Gly Gln Asn His His Glu Val  
 GCA CCC ATG GCA GAA GGA GGA GGG CAG AAT CAT CAC GAA GTG  
 20  
 277 Val Lys Phe Met Asp Val Tyr Gln Arg Ser Tyr Cys His Pro  
 GTG AAG TTC ATG GAT GTC TAT CAG CGC AGC TAC TGC CAT CCA  
 25  
 319 Ile Glu Thr Leu Val Asp Ile Phe Gln Glu Tyr Pro Asp Glu  
 ATC GAG ACC CTG GTG GAC ATC TTC CAG GAG TAC CCT GAT GAG  
 50  
 361 Ile Glu Tyr Ile Phe Lys Pro Ser Cys Val Pro Leu Met Arg  
 ATC GAG TAC ATC TTC AAG CCA TCC TGT GTG CCC CTG ATG CGA  
 60 70  
 403 Cys Gly Gly Cys Cys Asn Asp Glu Gly Leu Glu Cys Val Pro  
 TGC GGG GGC TGC TGC AAT GAC GAG GGC CTG GAG TGT GTG CCC  
 80  
 445 Thr Glu Glu Ser Asn Ile Thr Met Gln Ile Met Arg Ile Lys  
 ACT GAG GAG TCC AAC ATC ACC ATG CAG ATT ATG CGG ATC AAA  
 90  
 487 Pro His Gln Gly Gln His Ile Gly Glu Met Ser Phe Leu Gln  
 CCT CAC CAA GGC CAG CAC ATA GGA GAG ATG AGC TTC CTA CAG  
 100 110  
 529 His Asn Lys Cys Glu Cys Arg Pro Lys Lys Asp Arg Ala Arg  
 CAC AAC AAA TGT GAA TGC AGA CCA AAG AAA GAT AGA GCA AGA  
 571 Gln Glu Lys Lys Ser Val Arg Gly Lys Gly Lys Gly Gln Lys  
 CAA GAA AAA AAA TCA GTT CGA GGA AAG GGA AAG GGG CAA AAA  
 130 140  
 613 Arg Lys Arg Lys Lys Ser Arg Tyr Lys Ser Trp Ser Val Pro  
 CGA AAG CGC AAG AAA TGC CGG TAT AAG TCC TGG AGC GTT CCC

150  
 655 Cys Gly Pro Cys Ser Glu Arg Arg Lys His Leu Phe Val Gln  
 TGT GGG CCT TGC TCA GAG CGG AGA AAG CAT TTG TTT GTA CAA  
 5  
 160  
 697 Asp Pro Gln Thr Cys Lys Cys Ser Cys Lys Asn Thr Asp Ser  
 GAT CCG CAG ACG TGT AAA TGT TCC TGC AAA AAC ACA GAC TCG  
 10  
 170 180  
 739 Arg Cys Lys Ala Arg Gln Leu Glu Leu Asn Glu Arg Thr Cys  
 CGT TGC AAG GCG AGG CAG CTT GAG TTA AAC GAA CGT ACT TGC  
 15  
 781 Arg Cys Asp Lys Pro Arg Arg \*\*\*  
 AGA TGT GAC AAG CCG AGG CGG TGA GCCGGGCAGG AGGAAGGAGC  
 825 CTCCCTCAGG GTTTCGGGAA CCAGATCTCT CACCAGGAAA GACTGATACA  
 20  
 875 GAACGATCGA TACAGAAACC ACGCTGCCGC CACCACACCA TCACCATCGA  
 925 CAGAACAGTC CTTAATCCAG AAACCTGAAA TGAAGGAAGA GGAGACTCTG  
 975 CGCAGAGCAC TTTGGGTCCG GAGGGCGAGA CTCCGGCGGA AGCATTCCCG  
 25  
 1025 GGCGGGTGAC CCAGCACGGT CCCTCTTGGA ATTGGATTCTG CCATTTTATT  
 1075 TTTCTTGCTG CTAAATCACC GAGCCCGGAA GATTAGAGAG TTTTATTTCT  
 30  
 1125 GGGATTCCTG TAGACACACC CACCCACATA CATACTTTA TATATATATA  
 1175 TATTATATAT ATATAAATTA A

The gene coding for the hVPF of this invention can be cloned into and expressed in prokaryotic and eukaryotic  
 35 hosts. For example, active hVPF protein can be expressed in a prokaryotic host such as *E. coli* and a eukaryotic host  
 such as C-127 mouse cells or Chinese hamster ovary (CHO) cells by operably inserting the hVPF coding sequence  
 in replicable expression vectors or plasmids. For example, it can be inserted into a suitable plasmid such as pML  
 for production in *E. coli* and the bovine papilloma virus (BPV) vector for production in mouse cells or a shuttle vector which  
 can replicate in both prokaryotic and eukaryotic cells. In a preferred embodiment, the gene coding for the hVPF was  
 40 cloned into and expressed from C-127 mouse cells. The excreted protein was determined by the assay of Miles and  
 Miles, *supra*, (hereinafter also referred to as the Miles assay).

The original source material for the production of hVPF is the human histiocytic lymphoma cell line U-937. This  
 cell line was originally established from cells from the pleural effusion of a patient with diffuse histiocytic lymphoma as  
 reported by Sundstrom and Nilsson, *Int. J. Cancer* 17, 565-577 (1976). These cells are widely distributed as evidenced  
 45 by publications and are also readily available to the public in an unrestricted culture deposit from the American Type  
 Culture Collection, Rockville, Maryland, under accession number ATCC CRL 1593. Further background on these cells  
 can be found, for example, in *J. Exp. Med.* 143, 1528-1533 (1976), *Nature* 279, 328-331 (1979) and *J. Immunol.* 125,

4. Upon Assay: The human VPF activity was determined by the Miles assay. The results of the assay are not disclosed.

The human VPF produced by U-937 cells was originally identified by its inhibition and binding by rabbit polyclonal  
 antibodies to guinea pig VPF. A rabbit polyclonal antiserum to guinea pig VPF inhibited the permeability activity pro-  
 duced by U-937 cells as determined by the Miles assay. This U-937 generated VPF activity was about 70% to 80%  
 inhibited by the antiserum. The antiserum was also used to purify the VPF from the culture medium. A suitable process for the  
 production of human VPF comprises growing cells derived from the human histiocytic lymphoma cell line U-937 in serum-free nutrient culture medium at about 35° to 38°C for a sufficient time to elaborate

VPF and isolating the resulting VPF from the spent cells or the cell culture conditioned medium and isolating the human VPF from the cell culture conditioned medium of the U-937 cells described in said co-pending application by the following steps:

- 5 (a) cation exchange chromatography of said conditioned cell culture medium, for example with a column of CM-cellulose, CM-Sephadex®, Amberlite® IR-120H or S-Sepharose Fast Flow cation exchanger;
- (b) metal affinity chromatography of the VPF active fractions from said cation exchange chromatography, for example with a  $\text{Cu}^{2+}$ ,  $\text{Zn}^{2+}$  or  $\text{Ni}^{2+}$ /iminodiacetic acid(IDA)/Sepharose column; and
- 10 (c) reverse phase HPLC of the active VPF fractions from said method affinity chromatography, for example with a  $\text{C}_4$  or  $\text{C}_{18}$  reverse phase HPLC column.

15 The thus purified human VPF was found to be a protein of  $M_r$  34,000-42,000. When subjected to N-terminal amino-acid sequence analysis, it was found to have a distinct and novel structure whereby it differed from gVPF in four of the first ten amino acid positions.

The purified hVPF was active in promoting vessel leakage at a dose of 22 ng ( $5.5 \times 10^{-13}$  Moles) upon intradermal injection into guinea pigs. This highly purified hVPF thus is 9 times more potent than the gVPF described in U.S. Patent 4,456,550. Another advantage of hVPF of this invention is its human origin which is indicative of potential use as a human therapeutic compared to other agents of lesser purity or derived from guinea pig or other animals such as would cause immunological reactions.

#### Detailed Description of the Invention

25 While the specification concludes with claims particularly pointing out and distinctly claiming the subject matter regarded as forming the present invention, it is believed that the invention will be better understood from the following detailed description of preferred embodiments of the invention taken in conjunction with the accompanying drawings in which:

FIG. 1 is a schematic representation which shows the stepwise isolation of the human VPF (hVPF) from the conditioned cell culture medium of U-937 cells in one embodiment of the invention.

30 FIG. 2 is a graphical representation which shows the elution patterns in the stepwise purification of the hVPF in four panels A, B, C and D in the embodiment of FIG. 1 as follows:

35 A.) Cation Exchange Chromatography of hVPF. Serum-free conditioned medium from U-937 cells (6 L of 6-fold concentrate) was adjusted to pH 7.0 and loaded at a flow rate of 60 ml/hr onto an S-Sepharose column (5 x 45 cm) equilibrated in 0.01 M sodium phosphate, pH 7.0. A linear gradient from 0.2 M to 0.8 M NaCl in the same buffer was used to elute hVPF.

40 B.) Metal Affinity Chromatography of hVPF. The active eluate from the cation exchange column was concentrated to 20 ml by ultrafiltration and loaded onto a Sepharose (Fast Flow)/IDA/ $\text{Cu}^{+2}$  column equilibrated in 0.01 M sodium phosphate, pH 7.0, 2 M sodium chloride, 0.5 M imidazole. hVPF was eluted with a linear gradient of imidazole as shown.

45 C.) RP-HPLC of hVPF. The active eluate from the metal affinity column was loaded onto a  $\text{C}_{18}$  RP-HPLC column equilibrated with 0.05% trifluoroacetic acid (TFA) in water and eluted at 1 ml/min with a gradient of acetonitrile as shown.

Standards were first reduced with 2-mercaptoethanol.

FIG. 3 shows the ELISA of hVPF and gVPF. Rabbit anti-gVPF IgG was used to coat the polystyrene wells of microtiter plates. Various amounts of either hVPF (lower panel) or gVPF (upper panel) were allowed to bind overnight, and the amount of bound antigen detected with biotin-anti-gVPF IgG followed by HRP-avidin. Wells were developed with HRP substrate; the absorbance was read at 490 nm. Wells containing readings that were off scale were diluted and re-read in the linear range of the Bio-Tek microplate reader; the absorbances given are corrected for dilution. Different x-axis scales were used for hVPF and gVPF. Early and late bleed refers to both primary and secondary biotin-conjugated IgGs prepared from sera collected at the fourth bleed (after 3 immunizations) and at the eleventh bleed

(after 5 immunizations), respectively.

FIG. 4 shows the structure of plasmid hVPF/pUC9 of about 6200 bp. This plasmid, also designated pMON3044, contains the hVPF cDNA. The heavy arrow represents the location of the coding region for hVPF within the 3.5 kb cDNA.

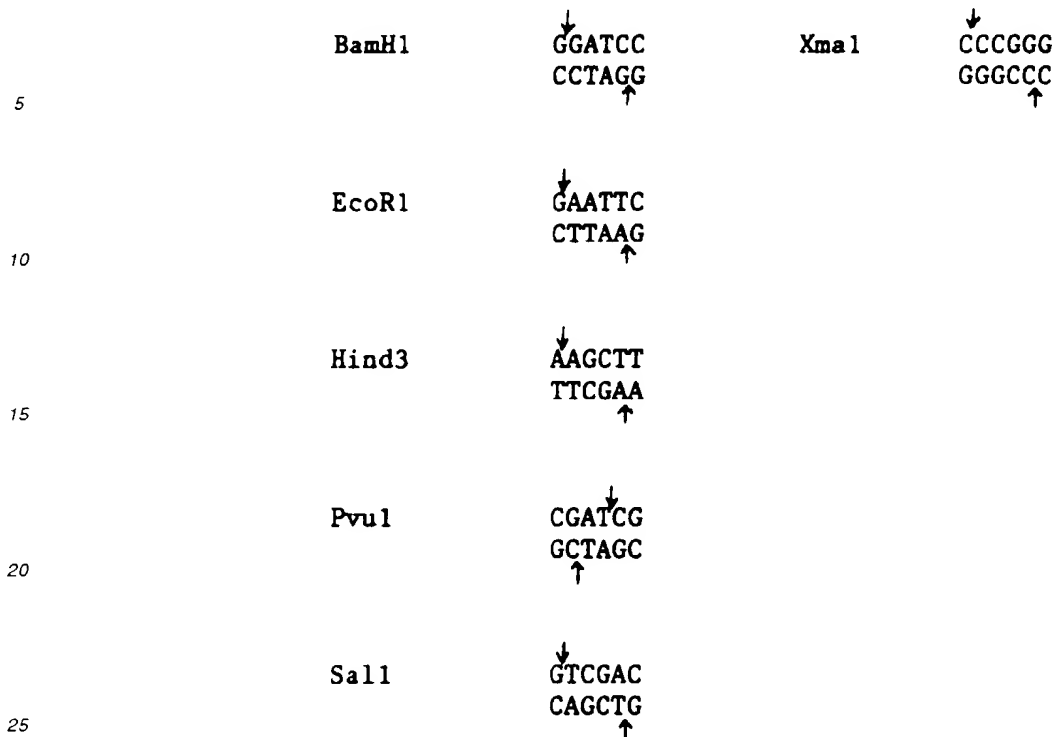
Plasmid pUC9 is a commercially available versatile vector that contains a multiple cloning site and a gene that confers ampicillin (AMP) resistance. The plasmid produces the  $\alpha$ -peptide of  $\beta$ -galactosidase which complements the lac deletion mutation in E. coli strains such as DH5a, JM83 and TB1.

Standard biochemical nomenclature is used herein in which the nucleotide bases of DNA or oligonucleotides are designated as adenine (A); thymine (T); guanine (G); and cytosine (C). N connotes any of these nucleotides. As is conventional for convenience in the structural representation of a DNA nucleotide sequence, only one strand is usually shown in which A on one strand connotes T on its complement and G connotes C. All sequences are written 5' to 3'. Amino acids are shown either by three letter or one letter abbreviations as follows:

Abbreviated Designation	Amino Acid
A Ala	Alanine
C Cys	Cysteine
D Asp	Aspartic acid
E Glu	Glutamic acid
F Phe	Phenylalanine
G Gly	Glycine
H His	Histidine
I Ile	Isoleucine
K Lys	Lysine
L Leu	Leucine
M Met	Methionine
N Asn	Asparagine
P Pro	Proline
Q Gln	Glutamine
R Arg	Arginine
S Ser	Serine
T Thr	Threonine
V Val	Valine
W Trp	Tryptophan
Y Tyr	Tyrosine

Commonly available restriction endonucleases shown in Example 4 and FIG. 4 have the following restriction sequences and (indicated by arrows) cleavage patterns.





The U-937 cells can be cultured in well-known cell culture media such as basal medium Eagle's (BME), Dulbecco's modified Eagle medium (DMEM), medium 199, RPMI 1640 medium, and the like cell culture media such as described in detail by H. J. Morton, *In Vitro* 6, 89-108 (1970). These conventional culture media contain known amino acids, mineral salts, vitamins, hormones and carbohydrates. They are also frequently fortified with mammalian sera such as fetal bovine serum (FBS). Other components which can be used in the media are bovine serum albumin (BSA), growth factors such as transferrin and insulin, protein hydrolysates such as lactalbumin hydrolysate, tryptone, tryptose and peptone, as well as lipids, surfactants and the like materials. The U-937 cells preferably are cultured in serum-free media for the production of hVPE.

Methods for the large scale growth of mammalian cells are well-known and these methods can be used for the culture of the U-937 cells defined herein. Such methods are described, for example, by Tolbert et al., *Biotech. Bioeng.* XXIV, 1671-1679 (1982); Tolbert and Feder, *Ann. Rept. Ferm. Proc.* Vol. 6, Ch. 3, pp. 35-74 (1983); Harakas, *Ibid.* Vol. 7, Ch. 7, pp. 159-211 (1984); and references cited in said publications. U.S. Pat. Nos. 4,166,768; 4,289,854; and 4,537,860 disclose particularly useful methods and apparatus for the large scale growth and maintenance of cells for the production of proteinaceous materials. The disclosures in said patents are incorporated herein by reference. The methods and apparatus disclosed therein can be used for the culture of the U-937 cells defined herein.

The cells also can be cultured on a large scale basis in nutrient medium at 37°C. in agitated suspension culture as described in U.S. Pat. No. 4,289,854 and, after a suitable growth period, can be maintained in the static maintenance reactor described in U.S. Pat. No. 4,537,860 in which the medium is supplemented with 0.5% lactalbumin hydrolysate.

Although purification of the hVPE from the spent culture media can employ various known procedures for the separation of proteins such as, for example, salt and solvent fractionation, adsorption with colloidal materials, gel filtration, ion exchange chromatography, affinity chromatography, immuno-affinity chromatography, electrophoresis and

Such treatment of the cells has provided for the production of hVPE in quantities of up to 200 to 500 ng/ml of conditioned medium. This is equivalent to or greater than the gVPE levels reported for the guinea pig cell Line 10.

The U-937 cells have been passaged through nude mice by injection of  $1 \times 10^7$  cells into the mouse peritoneum. Nude mice are an immunodeficient species in which human cells can grow and not be rejected. After about 3 to 4 weeks the mice were sacrificed and soft tumors were removed from the abdominal cavity. The tumors were mechanically dissociated into cells and these U-937 cells were again put into culture.

The following examples will further illustrate the invention.

Example 1MATERIALS AND METHODS

5        Growth of U-937 Cells U-937 cells originally obtained from the American Type Culture Collection (ATCC) were subcloned in soft agar and selected for fast growth. One of these clones was selected for scale-up, but other clones, and even uncloned ATCC cells, also produced hVPF. The serum-free medium used contained the following components: RPMI 1640, DME (high glucose), Ham's F12 in a 1:1:1 ratio; HEPES (25 mM, pH 7.10-7.15); glutathione (1 mM); ethanolamine (20  $\mu$ M); selenium (30 nM); NaHCO<sub>3</sub> (2 mM); CuSO<sub>4</sub> (5 nM); NH<sub>4</sub>VO<sub>3</sub> (5 nM); ZnSO<sub>4</sub> (0.5  $\mu$ M);  
 10       MnSO<sub>4</sub> (0.5 nM); FeSO<sub>4</sub> (4  $\mu$ M); bovine serum albumin, Miles "Pentex" (100  $\mu$ g/ml); iron rich transferrin, Miles (5  $\mu$ g/ml); bovine insulin (10  $\mu$ g/ml); ExCyte, Miles-lipid fraction (0.1% v/v); F-68 Pluracol® (0.05% w/v). The volume was adjusted to yield an osmolarity of 280 mOsm. The doubling time was about 50-60 hours in this medium, whereas it was only 35-40 hours in medium containing 2% fetal bovine serum (FBS).

Cells were scaled-up in the serum-free medium from T-flasks into roller bottles and then into small spinners. A 12  
 15       L spinner was then used to inoculate a 14 L perfusion chemostat which was perfused at a rate of approximately 3 ml medium/hour/ml of total packed cells. The culture was subsequently transferred to a 100 L perfusion chemostat, which was perfused under limiting nutrient conditions (1.5-2.0 ml medium/hour/ml packed cells, or 0.1-0.15 ml/day/million cells). Cells were recycled to the reactor using an AG Technology hollow fiber-cartridge (model CFP-4-E-6, 0.4 micron). Cell density ranged from 1.0 x 10<sup>6</sup> to 4.6 x 10<sup>6</sup> viable cells/ml, 3.0 to 23 ml/L of packed cells, and viability ranged from  
 20       64% to 84%. The production run in the 100 L reactor lasted 24 days, during which time a total of 1000 L of serum free conditioned medium was produced.

The permeate from the perfusion reactor was collected and stored at 4°C. Concentration was performed in 200-300 L lots on an Amicon DC-30 ultrafiltration apparatus with three low protein binding 10 kDa cutoff spiral cartridges (Amicon S10Y10) operated in parallel. A concentration of about 6 fold was achieved, including a phosphate buffered saline (PBS) wash of the cartridges and equipment that was pooled with the concentrate. The concentrate was stored at -20°C.  
 25       Vascular Permeability Assay A Miles-type permeability assay (Miles and Miles, supra) was used to detect hVPF. Hairless guinea pigs (IAF/HA-HO, Charles River, Wilmington MA) were anesthetized by inhalation of methoxyflurane (Metofane®, Pitman-Moore, Inc.). A 1 ml volume of 0.5% (w/v) Evan's blue dye (Sigma Chemical Co.) prepared in sterile saline for injection (Abbott Laboratories) was injected intracardially into the circulation. Samples for hVPF de-

30       termination were prepared at appropriate dilutions in saline or phosphate buffered saline, and 200  $\mu$ l volumes injected intradermally into sites on the back of the guinea pig. The presence of hVPF was indicated by an intense blue spot at the site of the injection where dye (bound to serum protein) had leaked from the circulation into the tissues.

Cation Exchange Chromatography Six liters of six-fold concentrated conditioned medium was adjusted to pH 7.0 with acetic acid and passed through a column (5 cm X 44 cm) of S Sepharose® Fast Flow (Pharmacia) cation exchange gel equilibrated with 0.01 M sodium phosphate, pH 7.0. At 4°C a significant portion of the permeability enhancing activity passed through the column, but at ambient temperature (25°C) 50 to 70% of the activity became bound to the column. This step was therefore regularly performed at room temperature. Sodium azide (0.01% w/v) was added to all buffers. Flow rates for loading and elution were 10 ml per minute. After loading, the column was washed with 900 ml of 0.01 M sodium phosphate, pH 7.0, and then eluted with a 2.3 L linear gradient containing from 0.2 M to 0.8 M sodium chloride in the same buffer. Between runs, the column was washed with 0.1 M sodium hydroxide before re-equilibrating with 0.01 M sodium phosphate, pH 7.0.  
 40       Metal Affinity Chromatography Metal affinity chromatography was performed using a copper-iminodiacetic acid complex covalently linked to agarose via a spacer arm. The gel was synthesized in two steps by first adding an epoxide-containing spacer arm to the agarose, and then reacting the activated gel with iminodiacetic acid.

45       Highly cross-linked agarose (Sepharose Fast Flow, Pharmacia) was repeatedly washed with distilled water to remove all buffers and preservatives and then dried by suction. About 100 g (100 ml) of this damp gel was suspended in 60 ml distilled water, and then 40 ml freshly prepared 2.5 M NaOH solution was added to the gently stirring agarose

and distilled water to remove the excess epoxide and base. The washed, suction-dried gel was then added to 100 ml of 0.1 M active epoxide groups per mL gel. The activated gel was stored in distilled water at 4°C and generally used within 24 hours of preparation.

About 100 ml (100 g) of the activated Sepharose Fast Flow was washed with distilled water, dried by suction, and suspended in 100 ml of 1.0 M Na<sub>2</sub>NH(CH<sub>2</sub>CO<sub>2</sub>)<sub>2</sub>·H<sub>2</sub>O solution, which was adjusted to pH 11.0. This mixture was gently stirred at 65°C for 24 hours, and then repeatedly washed with distilled water to remove excess ligand. The functionalized gel was stored in ethanol-water (25:75 v/v) at 4°C until ready for use. Titration with thioisulfate showed the absence of epoxide groups, so capping with ethanolamine was deemed unnecessary. To determine the metal binding capacity of the gel, 10 ml of suction-dried gel was saturated with excess 50 mM Cu(ClO<sub>4</sub>)<sub>2</sub> and then carefully

washed with distilled water. Finally, the bound copper was removed with an excess of 50 mM Na<sub>2</sub>H<sub>2</sub>EDTA. Using standardized copper-EDTA solutions for comparison, the copper content was photometrically determined to be 43 micromoles Cu per milliliter of damp, suction-dried gel.

Chromatography was performed in a glass column (1.0 x 13 cm.) containing 10 ml of gel. The gel was charged with a 50 mM solution of Cu(ClO<sub>4</sub>)<sub>2</sub>, pH = 4.5, and then saturated with a buffered imidazole solution (20 mM imidazole + 2 M NaCl + 50 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.0). After thoroughly washing with 1.0 M NaCl, the column was then equilibrated with the starting buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.0, 2 M NaCl, 0.5 mM imidazole). The fractions from the cation exchange step containing permeability enhancing activity were pooled and concentrated from a volume of about 700 ml to 20 ml using an Amicon YM5 membrane. Upon concentration, a protein precipitate usually formed that could be removed by filtration. The sample was applied to the column at ambient temperature (25°C) and eluted at a flow rate of 0.5 ml/min over 500 minutes with a linear gradient of imidazole (0.5 mM to 60 mM) in 50 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.0, 2 M NaCl.

Reverse-Phase HPLC. Reverse-phase HPLC (RP-HPLC) was performed using a 4.6 mm x 25 cm Vydac column (Separations Group) containing 5 µm packing with 330 angstrom pore size. The mobile phases were: "A", 0.05% trifluoroacetic acid (TFA) in water and "B", 0.05% TFA in acetonitrile. After loading the sample, the column was washed with "A" until the absorbance again reached baseline, and then eluted with the following linear gradients: 0% to 20% "B" over 20 minutes, 20% to 40% "B" over the next 80 minutes, and then 40% to 100% "B" over the next 20 minutes. All flow rates were 1 ml/min. Fractions were collected in siliconized glass tubes.

N-Terminal Amino Acid Sequence Analysis. Automated Edman degradation chemistry was used to determine N-terminal amino acid sequence. An Applied Biosystems, Inc., model 470A gas phase sequencer (Foster City, CA) was employed for the degradations [Hunkapiller, et al, Methods Enzymol. 91, 399-413 (1983)]. The respective PTH-amino acid derivatives were identified by RP-HPLC analysis in an on-line fashion employing an Applied Biosystems, Inc., Model 120A PTH Analyzer fitted with a Brownlee 2.1 mm I.D. PTH-C<sub>18</sub> column. Yields of PTH amino acids were determined by comparison with an external standard mixture. The average repetitive yield was calculated by linear regression analysis of the log pmolar yield versus cycle number plot.

Similarities between the obtained sequence and known sequences were investigated using the computer program FAST A [Pearson and Lipman, Proc. Natl. Acad. Sci. USA 85, 2444-2448 (1988)]. Similarity searches were also performed against the National Biomedical Research Foundation (NBRF) protein sequence data base [Sidney et al., Nucleic Acids Res. 16, 1869-1871 (1988), Release 17, June 1988] and the translated GENBANK DNA base [Bilofsky and Burks, Nucleic Acids Res. 16, 1861-1864 (1988), Release 56, June 1988].

Tryptic peptides were prepared from reduced and alkylated hVPF. hVPF (1 nmole) was dissolved in 100 µl of 0.5 M Tris HCl, pH 8.5, 6 M guanidine-HCl, 1 mM EDTA, and 5 mM dithiothreitol. The solution was incubated for 30 minutes at 37°C before the addition of sodium iodoacetate (to a final concentration of 5 mM) and incubation at 4°C overnight. After dialyzing against 2 M guanidine HCl, 0.01 M Tris-HCl, pH 8.5, and then 0.1 M ammonium bicarbonate, 1 µg of TPCK treated trypsin (Sigma Chemical Co., St. Louis, MO) was added and the solution incubated overnight at 37°C. Peptides were separated by RP-HPLC using a Nucleosil C<sub>18</sub>, 5 micron, 100 Å, 4.6 x 250 mm column (Macherey-Nagel, Inc.). The flow rate was 1 ml/min at room temperature. A linear gradient of 0 to 90% acetonitrile in 0.1% trifluoroacetic acid run over 270 minutes was used for elution.

Electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on 10-15% gradient polyacrylamide gels using a Pharmacia PhastSystem. The buffer systems and the silver staining protocol were those that were recommended by the manufacturer.

Antibodies and Immunoassays. Rabbit polyclonal antiserum to gVPF (designated F001) was prepared by immunizing a New Zealand White (NZW) rabbit with repeated injections of gVPF. The first injection was in Complete Freund's Adjuvant followed by boosts in Incomplete Freund's Adjuvant. gVPF was purified by preparative SDS-PAGE [Senger et al, Science 219 983-985 (1983)] following purification by the method of Senger et al. Fed. Proc. 46, 2102 (1987).

Sandwich enzyme linked immunosorbant assays (ELISA) for human and guinea pig VPF were performed as described below. The IgG fraction of F001 antiserum was purified using adsorption onto protein A-Sepharose. 500 ng/well of the IgG obtained from the 11th bleed serum was coated onto polystyrene microtiter plates for 3 hours. Then

concentration was 0.1 µg/ml of VPF. The amount of bound antigen was detected with bound well containing 100 µl of 11th bleed F001 antiserum, for 2 hours followed by 1:2000 dilution of horseradish peroxidase (HRP)avidin (Cappe Labs) for 90 minutes. Wells were developed with the HRP substrate o-phenylenediamine-2HCl plus H<sub>2</sub>O<sub>2</sub> and the absorbance was read at 490 nm in a BioTek reader.

It had been observed that the earlier bleeds of rabbit F001 contained a higher concentration and/or higher affinity antibodies cross reactive with hVPF compared to later bleeds. Therefore, an ELISA for hVPF was also performed with an early bleed (4th bleed) antiserum. It was performed under the same conditions as described above except that 1500 ng/well of a biotin-F001 IgG (4th bleed) was used to detect the amount of bound antigen.

## RESULTS

U-937 Permeability Enhancing Activity. The serum-free conditioned medium from U-937 cells in culture produced a positive response when tested in the Miles permeability assay. These results were unexpected since many cells do not produce a VPF-like activity as seen from Example 2, below. The Miles assay measures extravassation of Evan's blue dye-serum albumin complexes from the circulation after intradermal injection of a test material. However, the assay is non-specific and could measure positive response from a variety of substances, including, for example, histamine. It was therefore not known initially if the permeability enhancing activity produced by U-937 cells was related to guinea pig tumor VPF. To test this, the medium was mixed with an immunoabsorbent composed of protein A-Sepharose® and IgG obtained from polyclonal anti-sera against gVPF. Most, but not all, of the permeability enhancing activity present in the U-937 medium was adsorbed using this procedure, but not when control IgG obtained from rabbits not immunized with gVPF was used instead. Most of the permeability enhancing activity secreted into the medium of U-937 cells therefore appeared to be related to guinea pig tumor derived VPF. However, as discussed below, even though hVPF shares some immunocrossreactivity with gVPF, it is immunologically distinct from gVPF.

hVPF Purification. Initial attempts at purification of U-937 cell derived permeability enhancing activity employed the purification method previously used for gVPF as described by Senger et al, *Fed. Proc.* **46**, 2102 (1987). The application of this method, or minor modifications thereof, did not produce homogeneous protein from U-937 cell conditioned medium, even though the chromatographic behaviour of the permeability enhancing activity was very similar to that of gVPF. A novel purification method was therefore developed that incorporated cation exchange chromatography, metal affinity chromatography, and RP-HPLC (Fig. 1). In the first step, concentrated conditioned medium was passed over an S Sepharose cation exchange column (Fig. 2A). About 50-70% of the permeability enhancing activity was bound to the column at pH 7.0. The non-adsorbed activity was not characterized. The bound activity was eluted with a gradient of sodium chloride, and after concentration by ultrafiltration, loaded onto a metal affinity column (Fig. 2B). All detectable activity was tightly bound by the copper/IDA-Sepharose column, and was eluted after most of the other proteins in a gradient of imidazole. The final step utilized RP-HPLC (Fig. 2C) and resulted in elution of a group of  $M_r$  ~40 kDa proteins in the fractions associated with the peak of permeability enhancing activity (Fig. 2D). This method has been repeated numerous times and it reproducibly yielded  $M_r$  ~40 kDa protein of about 90% or greater purity as analyzed by SDS-PAGE with silver staining or by N-terminal sequence analysis. Approximately 1-2 µg of pure protein can be obtained per liter of U-937 cell conditioned medium.

### Dose Response of hVPF Induced

Permeability Enhancement. Different amounts of hVPF were tested in the Miles permeability assay. The lowest dilution producing a positive response was at a hVPF concentration of about 2.75 nM. This corresponds to an injection dose of 22 ng, or 0.55 picomoles of  $M_r$  40 kDa hVPF. This is equivalent to only one-ninth the 200 ng required for a similar response by the gVPF described in U.S. Patent 4,456,550.

Amino Acid Sequence of hVPF. hVPF was subjected to N-terminal amino acid sequence analysis (Table 1). Complete identity was observed between hVPF and the guinea pig tumor derived gVPF for the first 6 positions, but the sequence diverged for the next 4 amino acids sequenced. The identity is thus only 60% in this N-terminal region.

Table 1

Comparison of N-Terminal Sequences of hVPF and gVPF		
	Residue (pMole Yield)	
Cycle	hVPF	gVPF
1	Ala (627)	Ala (838)
2	Glu (65)	Glu (456)
3	Glu (65)	Glu (456)
4	Gly (55)	Gly (434)
5	Gly (76)	Glu (537)
6	Gly (150)	Gln (276)
7	Gly (150)	Gln (276)
8	Asn (29)	Pro (354)



10

### Sequence of hVPF Tryptic Peptides

1.5Peptide20

53 Phe Met Asp Val Tyr Gln Arg  
71 Ile Phe Lys Pro Ser Cys Val Pro Leu Met Arg  
93 Val Asp Ile Phe Gln Glu Tyr Pro Asp Glu Ile Glu Tyr  
1 5 10

30

35

### Example 2

45

Table 3

	Cell Line	Conditioned Media Concn. (a)	Miles Assay (units)(b)	Estimates of VPF Concn. (ng/ml) (c)
5	U-937(ATCC)			
10	Uncloned	1X	1	50
	U-937(ATCC)	1X	2-8	100-400
	Cloned (d)			
15	U-937(ATCC)			
	Uncloned-			
	Passaged through	1X	4-8	200-400
	Nude mouse			
20	Mnng HOS			
	Human osteogenic	10X	+++ (e)	30 (e)
	sarcoma			
25	JURKAT			
	Human acute			
	lymphoblastic	12.3X	1-2	4-8
	leukemia			
30	F2.11-2X			
	Human T cell-T cell	10X	0	<5
	hybridoma			
35	HFF			
	Neonatal human	26.6X	0	<2
	foreskin fibroblasts			
40	1MR 90			
	Human fetal			
	lung fibroblasts	26.6X	5	10
	HEK			
	Human embryonic	16.9X	4	10
	kidney			
45	8387-M15			
	Human sarcoma	36X	>16	>20

(a) Conditioned media were concentrated X-fold as indicated prior to testing by the Miles assay.

(b) Activity of sample = greatest dilution of sample at which blue spot was detectable.

1 unit (u) = the amount of VPF in a sample producing the smallest detectable blue spot discernible from control injections without VPF.

(c) Estimate of VPF concentration in the unconcentrated spent media in ng/ml based on 1 unit activity = 50 ng/ml, a relationship established with the guinea pig Line 10 VPF of U.S. Patent 4,456,550.

(d) Several clones, obtained by limiting dilution cloning procedures, produced activities in the 100-800 ng/ml range.

(e) This is an estimate based on the previously determined activity of Mnng HOS cells, since dilutions of the samples were not performed in this assay.

### Example 3

### Materials

All reagents and abbreviated designations, unless otherwise specified, are the same as described by Maniatis et al. *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1982.

### Methods

#### Oligonucleotide Screening of $\lambda$ gt10 Clontech library

Methods: Approximately 10<sup>5</sup>  $\lambda$ gt10 clones from a phage library were screened for clones that encode hVPF. HOS cells (Clontech, Palo Alto, CA) were screened using two best guess oligonucleotide probes. One probe (5'-3' 5'-CTGGCCGCCGCCCTCGGCCATAGGAGC-3') which was based on N-terminal amino acid sequencing (amino acids 1-10 of hVPF) hybridized to a single gt10 clone. A second probe (5'-GTGGACATCTTCCAGGAGTACCCCGACGA-

GATCGAGTAC-3') based on sequence information from a tryptic peptide corresponding to amino acids 33-45 of hVPF hybridized to seven clones containing inserts ranging in size from 0.8 to 3.5 kb. The screening procedure was that of Ullrich et al., *EMBO J.* **3**, 361-364 (1984), with the following modifications: purified yeast tRNA (Sigma Cat. No. R3001) (0.01 mg/ml) was used instead of salmon sperm and the sodium pyrophosphate and ATP were deleted from the hybridization solution. The largest of these clones, clone 4, which was 3.5 kb in size, was subcloned into pUC9 (New England Biolabs), thereby generating plasmid vector pMON3044 (see fig. 4) and 1195 bp of sequence was obtained using the general dideoxy-termination method of Sanger et al., *Proc. Natl. Acad. Sci. USA* **74**, 5463-5467 (1977), employing reagents obtained from United States Biochemicals. The sequence corresponding to nucleotides 288 to 1110 was obtained by sequencing both strands of DNA. Nucleotides 147-288 were determined from only one strand, but were sequenced twice using two different sequencing primers.

DNA band migration patterns on agarose gels as a result of multiple restriction endonuclease digestion patterns of the pMON3044 construct indicated the orientation of the clone as shown in Fig. 4.

## RESULTS

A 3.5 Kb cDNA clone (designated VPF-4) was isolated from a  $\lambda$ gt10 library prepared from mRNA isolated from phorbol-ester stimulated U-937 cells. This clone was transferred to the plasmid vector pUC9 to thereby form construct pMON3044. Then 1195 bp of sequence for the hVPF cDNA was generated. A variety of restriction endonucleases were used to determine the orientation of the cDNA for hVPF in the plasmid vector. Subjecting the cDNA to the selected endonuclease treatment demonstrated that approximately 1300 and 1600 bp of untranslated nucleotide sequences for the amino and carboxy termini, respectively, exist in the hVPF cDNA. The size of the hVPF/pUC9 construct (see FIG. 4) is approximately 6200 bp. This is the sum of approximately 3500 bp from the hVPF cDNA and approximately 2700 bp from the plasmid pUC9.

The predicted cDNA sequence demonstrated 100% homology with the N-terminus and several tryptic peptides previously determined using direct amino acid sequencing. These sequences correspond to amino acids 1-18, 17-23, 33-45 and 46-55, respectively. A signal sequence (amino acids -26 to -1) and a single N-linked glycosylation site at Asn 75 were also found. These results confirm that hVPF is a secreted and glycosylated protein.

A further comparison between the amino acid sequences of hVPF and gVPF was made by comparing the N-terminal sequence of the U-937 cell-derived hVPF as shown above and the sequence of gVPF determined by amino acid sequencing of purified gVPF from guinea pig Line 10 tumor cells as follows:

hVPF	Ala	Pro	Met	Ala	Glu	Gly	Gly	Gly	Gln	Asn	His	His	Glu	Val	Val
	*	*	*	*	*	*			*				*	*	*
gVPF	Ala	Pro	Met	Ala	Glu	Gly	Glu	---	Gln	Lys	Pro	Arg	Glu	Val	Val
hVPF	Lys	Phe	Met	Asp	Val	Tyr	Gln	Arg	Ser	Tyr	Cys	His	Pro	Ile	Glu
	*	*	*	*	*	*		*	*	*	*		*	*	*
gVPF	Lys	Phe	Met	Asp	Val	Tyr	Lys	Arg	Ser	Tyr	Cys	Arg	Pro	Ile	Glu
hVPF	Thr	Leu	Val	Asp	Ile	Phe	Gln								
gVPF	Thr	Leu	Val	Asp	Ile	Phe	Gln								

Comparison of the amino termini of VPF from guinea pig and human tumor cell lines demonstrate distinct differences. A gap between amino acid positions 7 and 8 in the gVPF sequence must be inserted for overlap to occur between the two proteins. Additionally, using numbering from hVPF, several areas of nonidentical residues are noted and are indicated by asterisks in the above comparison.



Example 4Expression of Permeability-Enhancing

5        Activity by C-127 Cells Transfected with the hVPF Gene. Serum-free conditioned medium was collected from three selected hVPF cell lines (VPF-25B, -29B, and -30B) transfected with a BPV expression vector containing the hVPF cDNA sequence, or from a control line (BPV-1123) transfected with the BPV vector only. This BPV vector contains the entire bovine papilloma virus genome ligated to the well-known pBR322 derivative of pML2. Medium was concentrated 5-fold using a Centricon-10 ultrafiltration device before performing the Miles permeability assay. A positive response  
10        was indicated by the appearance of a blue spot at the site of intradermal injection of sample into the guinea pig, indicating extravasation of Evan's blue dye from the circulation.

      The hVPF cDNA was thus expressed in mammalian cells with a bovine papilloma virus (BPV) vector. This vector is based on the 100% viral genome and utilizes the mouse metallothionein I promoter and the SV40 Late poly A addition site to regulate the expression of foreign genes. The vector was linearized at the unique BamHI site between the  
15        promoter and poly A addition site. The 5' overhanging ends of the vector fragment were filled in with Klenow enzyme and dNTP's. Similarly, the plasmid containing the hVPF cDNA was digested with XmaI and the 1021 bp VPF fragment was isolated by gel electrophoresis. This blunt end fragment was then inserted into the vector by ligation. Mouse C-127 cells were cotransfected with the hVPF expression vector and pSV2neo by the calcium phosphate precipitation method as described by Howley et al., Meth. Enzymol. 101, 387-403 (1983). G418 (gentamicin) resistant transfectants  
20        were selected. Colonies were picked and expanded into stable lines for assay by the Miles assay.

**Claims**

25

**Claims for the following Contracting States : AT, BE, CH, DE, FR, GB, GR, IT, LI, LU, NL, SE**

1. Human vascular permeability factor having an amino acid sequence as follows:

30

Ala Pro Met Ala Glu Gly Gly Gly Gln Asn His His Glu Val

Val Lys Phe Met Asp Val Tyr Gln Arg Ser Tyr Cys His Pro

Ile Glu Thr Leu Val Asp Ile Phe Gln Glu Tyr Pro Asp Glu

35

Ile Glu Tyr Ile Phe Lys Pro Ser Cys Val Pro Leu Met Arg

Cys Gly Gly Cys Cys Asn Asp Glu Gly Leu Glu Cys Val Pro

40

Thr Glu Glu Ser Asn Ile Thr Met Gln Ile Met Arg Ile Lys

Pro His Gln Gly Gln His Ile Gly Glu Met Ser Phe Leu Gln

His Asn Lys Cys Glu Cys Arg Pro Lys Lys Asp Arg Ala Arg

45

Gln Glu Lys Lys Ser Val Arg Gly Lys Gly Lys Gly Gln Lys

Asp Pro Gln Thr Cys Lys Cys Ser Cys Lys Asn Thr Asp Ser

Arg Cys Lys Ala Arg Gln Leu Glu Leu Asn Glu Arg Thr Cys

Arg Cys Asp Lys Pro Arg Arg.

2. Human vascular permeability factor of Claim 1 which is N-glycosylated at amino acid residue Asn 75

3. Human vascular permeability factor of Claim 1 which is non-glycosylated.
4. Human vascular permeability factor of Claim 1 including a signal sequence at the N-terminus as follows:

Met Asn Phe Leu Leu Ser Trp Val His Trp Ser Leu

-10

Ala Leu Leu Leu Tyr Leu His His Ala Lys Trp Ser Gln Ala.

5. The cDNA of human vascular permeability factor including a nucleotide sequence substantially as follows:

151	ATG AAC TTT CTG CTG TCT TGG GTG CAT TGG AGC CTT
193	GCC TTG CTG CTC TAC CTC CAC CAT GCC AAG TGG TCC CAG GCT
235	GCA CCC ATG GCA GAA GGA GGA GGG CAG AAT CAT CAC GAA GTG
277	GTG AAG TTC ATG GAT GTC TAT CAG CGC AGC TAC TGC CAT CCA
319	ATC GAG ACC CTG GTG GAC ATC TTC CAG GAG TAC CCT GAT GAG
361	ATC GAG TAC ATC TTC AAG CCA TCC TGT GTG CCC CTG ATG CGA
403	TGC GGG GGC TGC TGC AAT GAC GAG GGC CTG GAG TGT GTG CCC
445	ACT GAG GAG TCC AAC ATC ACC ATG CAG ATT ATG CGG ATC AAA
487	CCT CAC CAA GGC CAG CAC ATA GGA GAG ATG AGC TTC CTA CAG
529	CAC AAC AAA TGT GAA TGC AGA CCA AAG AAA GAT AGA GCA AGA
571	CAA GAA AAA AAA TCA GTT CGA GGA AAG GGA AAG GGG CAA AAA
613	CGA AAG CGC AAG AAA TCC CGG TAT AAG TCC TGG AGC GTT CCC

655 TGT GGG CCT TGC TCA GAG CGG AGA AAG CAT TTG TTT GTA CAA  
697 GAT CCG CAG ACG TGT AAA TGT TCC TGC AAA AAC ACA GAC TCG  
739 CGT TGC AAG GCG AGG CAG CTT GAG TTA AAC GAA CGT ACT TGC  
781 AGA TGT GAC AAG CCG AGG CGG

781 AGA TGT GAC AAG CCG AGG CGG

6. Plasmid hVPF/pUC9 as shown in Figure 4 comprising the cDNA of the human vascular permeability factor as

8. Mammalian cells transfected with a vector containing the cDNA sequence for human vascular permeability factor of claim 5
9. A method of producing human vascular permeability factor comprising culturing the cells of claim 8
10. A method of producing human vascular permeability factor comprising culturing the cells of claim 8 or claim 9

11. Method for the production of human vascular permeability factor in vitro comprising growing cells derived from the human histiocytic lymphoma cell line U-937 in nutrient culture medium at about 35° to 38°C for a sufficient time to elaborate vascular permeability factor and isolating the resulting vascular permeability factor from the spent cells or the cell culture conditioned medium wherein the vascular permeability factor is isolated from the cell culture conditioned medium by

(a) cation exchange chromatography of said conditioned cell culture medium;

(b) metal affinity chromatography of the VPF active fractions from said cation exchange chromatography; and

(c) reverse phase HPLC of the active VPF fractions from said metal affinity chromatography.

12. The method of claim 11 in which the nutrient culture medium is serum-free.

13. The method of claim 11 in which the cation exchange chromatography is carried out with a column of S-Sepharose Fast Flow cation exchange, the metal affinity chromatography is carried out with a copper/immunodiacetic acid (IDA)/Sepharose column, and the reverse phase HPLC is carried out with a C<sub>18</sub> reverse phase HPLC column.

14. A human vascular permeability factor having a molecular weight of about 34 to 42 kDa as determined by non-reduced SDS-PAGE and an N-terminal amino acid sequence as follows:

<sup>1</sup>Ala-Pro-Met-Ala-Glu-Gly-Gly-Gly-Gln-Asn.<sup>10</sup>

15. The human VPF of Claim 14 containing the following internal sequences :

(a) (Gln)Gln Gln Lys Pro,

(b) (Arg)Gln Glu Gln Arg(Pro Lys),

(c) Phe Met Asp Val Tyr Gln Arg(Arg),

(d) Ile Phe Lys Pro Ser Cys Val Pro Leu Met Arg, and

(e) Val Asp Ile Phe Gln Glu Tyr Pro Asp Glu Ile Glu Tyr.

#### Claims for the following Contracting State : ES

1. A process which comprises producing human vascular permeability factor having an amino acid sequence as follows:



- 5

10

15

- 20

25

40

- 45

151           ATG AAC TTT CTG CTG TCT TGG GTG CAT TGG AGC CTT  
 193   GCC TTG CTG CTC TAC CTC CAC CAT GCC AAG TGG TCC CAG GCT  
 235   GCA CCC ATG GCA GAA GGA GGA GGG CAG AAT CAT CAC GAA GTG  
 277   GTG AAG TTC ATG GAT GTC TAT CAG CGC AGC TAC TGC CAT CCA  
 319   ATC GAG ACC CTG GTG GAC ATC TTC CAG GAG TAC CCT GAT GAG  
 361   ATC GAG TAC ATC TTC AAG CCA TCC TGT GTG CCC CTG ATG CGA  
 403   TGC GGG GGC TGC TGC AAT GAC GAG GGC CTG GAG TGT GTG CCC  
 445   ACT GAG GAG TCC AAC ATC ACC ATG CAG ATT ATG CGG ATC AAA  
 487   CCT CAC CAA GGC CAG CAC ATA GGA GAG ATG AGC TTC CTA CAG  
 529   CAC AAC AAA TGT GAA TGC AGA CCA AAG AAA GAT AGA GCA AGA  
 571   CAA GAA AAA AAA TCA GTT CGA GGA AAG GGA AAG GGG CAA AAA  
 613   CGA AAG CGC AAG AAA TCC CGG TAT AAG TCC TGG AGC GTT CCG  
 655   TGT GGG CCT TGC TCA GAG CGG AGA AAG CAT TTG TTT GTA CAA  
 697   GAT CCG CAG ACG TGT AAA TGT TCC TGC AAA AAC ACA GAC TCG  
 739   CGT TGC AAG GCG AGG CAG CTT GAG TTA AAC GAA CGT ACT TGC  
 781   AGA TGT GAC AAG CCG AGG CGG

6. A process which comprises producing plasmid hVPF/pUC9 as shown in Figure 4 comprising the cDNA of the human vascular permeability factor as defined in claim 5.
7. A process which comprises producing a human vascular permeability factor cDNA clone of about 3.5 kb including the cDNA of Claim 5.
8. Mammalian cells transfected with a vector containing the cDNA sequence for human vascular permeability factor of claim 5.
9. C-127 mouse cells transfected with a vector containing the cDNA sequence for human vascular permeability factor of claim 5.
11. Method for the production of human vascular permeability factor in vitro comprising growing cells derived from the human histiocytic lymphoma cell line U-937 in nutrient culture medium at about 35° to 38°C for a sufficient time to elaborate vascular permeability factor and isolating the resulting vascular permeability factor from the spent culture medium by ultrafiltration and ion exchange chromatography of said conditioned cell culture medium.

- (b) metal affinity chromatography of the VPF active fractions from said cation exchange chromatography; and  
(c) reverse phase HPLC of the active VPF fractions from said metal affinity chromatography.

5 12. The method of claim 11 in which the nutrient culture medium is serum-free.

13. The method of claim 11 in which the cation exchange chromatography is carried out with a column of S-Sepharose Fast Flow cation exchange, the metal affinity chromatography is carried out with a copper/immunodiacetic acid (IDA)/Sepharose column, and the reverse phase HPLC is carried out with a C<sub>18</sub> reverse phase HPLC column.

10 14. A process which comprises producing a human vascular permeability factor having a molecular weight of about 34 to 42 kDa as determined by non-reduced SDS-PAGE and an N-terminal amino acid sequence as follows:

15 <sup>1</sup>Ala-Pro-Met-Ala-Glu-Gly-Gly-Gly-Gln-Asn<sup>10</sup>.

15. The process of claim 14 wherein the human VPF contains the following internal sequences:

- 20 (a) (Gln)Gln Gln Lys Pro,  
(b) (Arg)Gln Glu Gln Arg(Pro Lys),  
(c) Phe Met Asp Val Tyr Gln Arg(Arg),  
(d) Ile Phe Lys Pro Ser Cys Val Pro Leu Met Arg, and  
(e) Val Asp Ile Phe Gln Glu Tyr Pro Asp Glu Ile Glu Tyr.

25

# Patentansprüche

30 Patentansprüche für folgende Vertragsstaaten : AT, BE, CH, LI, DE, FR, GB, GR, IT, LU, NL, SE

1. Menschlicher Gefäßpermeabilitätsfaktor (VPF) mit nachstehender Aminosäuresequenz:

35

40

45

Ala Pro Met Ala Glu Gly Gly Gly Gln Asn His His Glu Val  
 Val Lys Phe Met Asp Val Tyr Gln Arg Ser Tyr Cys His Pro  
 5 Ile Glu Thr Leu Val Asp Ile Phe Gln Glu Tyr Pro Asp Glu  
 Ile Glu Tyr Ile Phe Lys Pro Ser Cys Val Pro Leu Met Arg  
 10 Cys Gly Gly Cys Cys Asn Asp Glu Gly Leu Glu Cys Val Pro  
 Thr Glu Glu Ser Asn Ile Thr Met Gln Ile Met Arg Ile Lys  
 15 Pro His Gln Gly Gln His Ile Gly Glu Met Ser Phe Leu Gln  
 His Asn Lys Cys Glu Cys Arg Pro Lys Lys Asp Arg Ala Arg  
 Gln Glu Lys Lys Ser Val Arg Gly Lys Gly Lys Gly Gln Lys  
 20 Arg Lys Arg Lys Lys Ser Arg Tyr Lys Ser Trp Ser Val Pro  
 Cys Gly Pro Cys Ser Glu Arg Arg Lys His Leu Phe Val Gln  
 25 Asp Pro Gln Thr Cys Lys Cys Ser Cys Lys Asn Thr Asp Ser  
 Arg Cys Lys Ala Arg Gln Leu Glu Leu Asn Glu Arg Thr Cys  
 Arg Cys Asp Lys Pro Arg Arg.

30

2. Menschlicher Gefäßpermeabilitätsfaktor nach Anspruch 1, der am Aminosäurerest Asn 75 N-glykosyliert ist.
3. Menschlicher Gefäßpermeabilitätsfaktor nach Anspruch 1, der nichtglykosyliert ist.
4. Menschlicher Gefäßpermeabilitätsfaktor nach Anspruch 1, umfassend nachstehende Signalsequenz am N-Terminus:

35

40

<sup>-26</sup> Met Asn Phe Leu Leu Ser <sup>-20</sup> Trp Val His Trp Ser Leu

45

<sup>-10</sup> Ala Leu Leu Leu Tyr Leu His His Ala Lys Trp Ser Gln Ala.

5. cDNA des menschlichen Gefäßpermeabilitätsfaktors, umfassend eine Nukleotidsequenz im wesentlichen wie

151           ATG AAC TTT CTG CTG TCT TGG GTG CAT TGG AGC CTT  
 193   GCC TTG CTG CTC TAC CTC CAC CAT GCC AAG TGG TCC CAG GCT  
 235   GCA CCC ATG GCA GAA GGA GGA GGG CAG AAT CAT CAC GAA GTG  
 277   GTG AAG TTC ATG GAT GTC TAT CAG CGC AGC TAC TGC CAT CCA  
 319   ATC GAG ACC CTG GTG GAC ATC TTC CAG GAG TAC CCT GAT GAG  
 361   ATC GAG TAC ATC TTC AAG CCA TCC TGT GTG CCC CTG ATG CGA  
 403   TGC GGG GGC TGC TGC AAT GAC GAG GGC CTG GAG TGT GTG CCC  
 445   ACT GAG GAG TCC AAC ATC ACC ATG CAG ATT ATG CGG ATC AAA  
 487   CCT CAC CAA GGC CAG CAC ATA GGA GAG ATG AGC TTC CTA CAG  
 529   CAC AAC AAA TGT GAA TGC AGA CCA AAG AAA GAT AGA GCA AGA  
 571   CAA GAA AAA AAA TCA GTT CGA GGA AAG GGA AAG GGG CAA AAA  
 613   CGA AAG CGC AAG AAA TCC CGG TAT AAG TCC TGG AGC GTT CCC  
 655   TGT GGG CCT TGC TCA GAG CGG AGA AAG CAT TTG TTT GTA CAA  
 697   GAT CCG CAG ACG TGT AAA TGT TCC TGC AAA AAC ACA GAC TCG  
 739   CGT TGC AAG GCG AGG CAG CTT GAG TTA AAC GAA CGT ACT TGC  
 781   AGA TGT GAC AAG CCG AGG CGG

6. Plasmid hVPF/pUC9, das in Fig.4 dargestellt ist, umfassend die cDNA des menschlichen Gefäßpermeabilitätsfaktor nach Anspruch 5.
7. Menschlicher Gefäßpermeabilitätsfaktor-cDNA-Klon von etwa 3,5 kb, enthaltend die cDNA nach Anspruch 5.
8. Säugetierzellen, die mit einem Vektor transfiziert sind, der die cDNA-Sequenz für menschlichen Gefäßpermeabilitätsfaktor nach Anspruch 5 enthält.
9. C-127-Mäusezellen, die mit einem Vektor transfiziert sind, der die cDNA-Sequenz für menschlichen Gefäßpermeabilitätsfaktor nach Anspruch 5 enthält.
10. Verfahren zur Herstellung von menschlichem Gefäßpermeabilitätsfaktor, umfassend das Kultivieren der Zellen

a) in einem Medium, das eine geeignete Zellkulturmedium enthält, um einen ausreichenden Zeitraum, um Gefäßpermeabilitätsfaktor zu entwickeln, und das Isolieren des resultierenden Gefäßpermeabilitätsfaktors aus den erschöpften Zellen oder dem konditionierten Zellkulturmedium, worin der Gefäßpermeabilitätsfaktor durch folgende Schritte aus dem konditionierten Zellkulturmedium isoliert wird

i) Extrahieren des konditionierten Zellkulturmediums

ii) Metallaffinitätschromatographie der aktiven VPF-Fractionen aus der Kationenaustauschchromatographie und



(c) Reverse-Phase-HPLC der aktiven VPF-Fractionen aus der Metallaffinitätschromatographie.

12. Verfahren nach Anspruch 11, worin das Nährstoffkulturmedium serumfrei ist.

5 13. Verfahren nach Anspruch 11, worin die Kationenaustauschchromatographie mit einer S-Sepharose Fast Flow-Kationenaustausch-Säule erfolgt, die Metallaffinitätschromatographie mit einer Kupfer/Immediessigsäure-(IDA)/Sepharose-Säule erfolgt und die Reverse-Phase-HPLC mit einer C<sub>18</sub>-Reverse-Phase-HPLC-Säule erfolgt.

10 14. Menschlicher Gefäßpermeabilitätsfaktor mit einem Molekulargewicht von etwa 34 bis 42 kDa (bestimmt durch nichtreduzierte SDS-PAGE) und nachstehender N-terminaler Aminosäuresequenz:

<sup>1</sup> Ala-Pro-Met-Ala-Glu-Gly-Gly-Gln-Asn. <sup>10</sup>

15

15. Menschlicher VPF nach Anspruch 14, umfassend die folgenden internen Sequenzen:

- 20 (a) (Gln)Gln Gln Lys Pro,  
(b) (Arg)Gln Glu Gln Arg(Pro Lys),  
(c) Phe Met Asp Val Tyr Gln Arg(Arg),  
(d) Ile Phe Lys Pro Ser Cys Val Pro Leu Met Arg, and  
(e) Val Asp Ile Phe Gln Glu Tyr Pro Asp Glu Ile Glu Tyr.

25 **Patentansprüche für folgenden Vertragsstaat : ES**

1. Verfahren, umfassend die Herstellung von menschlichem Gefäßpermeabilitätsfaktor mit nachstehender Aminosäuresequenz:

30

Ala Pro Met Ala Glu Gly Gly Gly Gln Asn His His Glu Val

Val Lys Phe Met Asp Val Tyr Gln Arg Ser Tyr Cys His Pro

Ile Glu Thr Leu Val Asp Ile Phe Gln Glu Tyr Pro Asp Glu

35

Ile Glu Tyr Ile Phe Lys Pro Ser Cys Val Pro Leu Met Arg

Cys Gly Gly Cys Cys Asn Asp Glu Gly Leu Glu Cys Val Pro

40

Thr Glu Glu Ser Asn Ile Thr Met Gln Ile Met Arg Ile Lys

Pro His Gln Gly Gln His Ile Gly Glu Met Ser Phe Leu Gln

His Asn Lys Cys Glu Cys Arg Pro Lys Lys Asp Arg Ala Arg

45

Gln Glu Lys Lys Ser Val Arg Gly Lys Gly Lys Gly Gln Lys

Lys Gly Leu Lys Ser Ile Arg Arg Lys His Leu Phe Val Gln

Asp Pro Gln Thr Cys Lys Cys Ser Cys Lys Asn Thr Asp Ser

Arg Cys Lys Ala Arg Gln Leu Gln Leu Asn Glu Arg Thr Cys

Arg Cys Asp Lys Pro Arg Arg.

2. Verfahren nach Anspruch 1, worin der menschliche Gefäßpermeabilitätsfaktor an Aminosäurerest Asn 75 N-glykosyliert ist.
3. Verfahren nach Anspruch 1, worin der menschliche Gefäßpermeabilitätsfaktor nichtglykosyliert ist.
4. Verfahren nach Anspruch 1, worin der menschliche Gefäßpermeabilitätsfaktor nachstehende Signalsequenz am N-Terminus umfaßt:

-16                      -20  
Met Asn Phe Leu Leu Ser Trp Val His Trp Ser Leu

-10

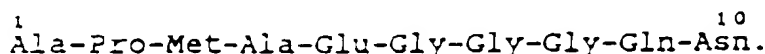
Ala Leu Leu Leu Tyr Leu His His Ala Lys Trp Ser Gln Ala.

5. Verfahren, umfassend die Herstellung der cDNA des menschlichen Gefäßpermeabilitätsfaktors, der eine Nukleotidsequenz im wesentlichen wie folgt enthält:

151	ATG AAC TTT CTG CTG TCT TGG GTG CAT TGG AGC CTT
193	GCC TTG CTG CTC TAC CTC CAC CAT GCC AAG TGG TCC CAG GCT
235	GCA CCC ATG GCA GAA GGA GGA GGG CAG AAT CAT CAC GAA GTG
277	GTG AAG TTC ATG GAT GTC TAT CAG CGC AGC TAC TGC CAT CCA
319	ATC GAG ACC CTG GTG GAC ATC TTC CAG GAG TAC CCT GAT GAG
361	ATC GAG TAC ATC TTC AAG CCA TCC TGT GTG CCC CTG ATG CGA
403	TGC GGG GGC TGC TGC AAT GAC GAG GGC CTG GAG TGT GTG CCC
445	ACT GAG GAG TCC AAC ATC ACC ATG CAG ATT ATG CGG ATC AAA
487	CCT CAC CAA GGC CAG CAC ATA GGA GAG ATG AGC TTC CTA CAG
529	CAC AAC AAA TGT GAA TGC AGA CCA AAG AAA GAT AGA GCA AGA
571	CAA GAA AAA AAA TCA GTT CGA GGA AAG GGA AAG GGG CAA AAA
613	CGA AAG CGC AAG AAA TCC CGG TAT AAG TCC TGG AGC GTT CCC
655	TGT GGG CCT TCC TCA GAG CGG AGA AAG CAT TTG TTT GTA CAA
697	GAT CCG CAG ACG TGT AAA TGT TCC TGC AAA AAC ACA GAC TCG
739	CGT TGC AAG GCG AGG CAG CTT GAG TTA AAC GAA CGT ACT TGC
781	AAA TGT CAC AAT GCG AGG CGG

7. Verfahren, umfassend die Herstellung eines menschlichen Gefäßpermeabilitätsfaktor-cDNA-Klons von etwa 3.5 kb, wobei die cDNA nach Anspruch 5 enthält
8. Saugtierzellen, die mit einem Vektortransfiziert sind, der die cDNA nach Anspruch 5 enthält

9. C-127-Mäusezellen, die mit einem Vektor transfiziert sind, der die cDNA-Sequenz für menschlichen Gefäßpermeabilitätsfaktor nach Anspruch 5 enthält.
10. Verfahren zur Herstellung von menschlichem Gefäßpermeabilitätsfaktor, umfassend das Kultivieren der Zellen nach Anspruch 8 oder 9.
11. Verfahren zur Herstellung von menschlichem Gefäßpermeabilitätsfaktor in vitro, umfassend das Züchten von Zellen aus der menschlichen Histiocytenlymphom-Zelllinie U-937 in Nährstoffkulturmedium bei etwa 35° bis 38°C über einen ausreichenden Zeitraum, um Gefäßpermeabilitätsfaktor zu entwickeln, und das Isolieren des resultierenden Gefäßpermeabilitätsfaktors aus den erschöpften Zellen oder dem konditionierten Zellkulturmedium, worin der Gefäßpermeabilitätsfaktor durch folgende Schritte aus dem konditionierten Zellkulturmedium isoliert wird:
- (a) Kationenaustauschchromatographie des konditionierten Zellkulturmediums;
  - (b) Metallaffinitätschromatographie der aktiven VPF-Fractionen aus der Kationenaustauschchromatographie; und
  - (c) Reverse-Phase-HPLC der aktiven VPF-Fractionen aus der Metallaffinitätschromatographie.
12. Verfahren nach Anspruch 11, worin das Nährstoffkulturmedium serumfrei ist.
13. Verfahren nach Anspruch 11, worin die Kationenaustauschchromatographie mit einer S-Sepharose Fast Flow-Kationenaustausch-Säule erfolgt, die Metallaffinitätschromatographie mit einer Kupfer/Immundiessigsäure-(IDA)/Sepharose-Säule erfolgt und die Reverse-Phase-HPLC mit einer C<sub>18</sub>-Reverse-Phase-HPLC-Säule erfolgt.
14. Verfahren, umfassend die Herstellung eines menschlichen Gefäßpermeabilitätsfaktors mit einem Molekulargewicht von etwa 34 bis 42 kDa (bestimmt durch nichtreduzierte SDS-PAGE) und nachstehender N-terminaler Aminosäuresequenz:



15. Verfahren nach Anspruch 14, worin der menschliche VPF die folgenden internen Sequenzen enthält:
- (a) (Gln)Gln Gln Lys Pro,
  - (b) (Arg)Gln Glu Gln Arg(Pro Lys),
  - (c) Phe Met Asp Val Tyr Gln Arg(Arg),
  - (d) Ile Phe Lys Pro Ser Cys Val Pro Leu Met Arg, and
  - (e) Val Asp Ile Phe Gln Glu Tyr Pro Asp Glu Ile Glu Tyr.

# Revendications

Revendications pour les Etats contractants suivants : AT, BE, CH, LI, DE, FR, GB, GR, IT, LU, NL, SE

Ala Pro Met Ala Glu Gly Gly Gly Gln Asn His His Glu Val  
 Val Lys Phe Met Asp Val Tyr Gln Arg Ser Tyr Cys His Pro  
 5 Ile Glu Thr Leu Val Asp Ile Phe Gln Glu Tyr Pro Asp Glu  
 Ile Glu Tyr Ile Phe Lys Pro Ser Cys Val Pro Leu Met Arg  
 10 Cys Gly Gly Cys Cys Asn Asp Glu Gly Leu Glu Cys Val Pro  
 Thr Glu Glu Ser Asn Ile Thr Met Gln Ile Met Arg Ile Lys  
 Pro His Gln Gly Gln His Ile Gly Glu Met Ser Phe Leu Gln  
 15 His Asn Lys Cys Glu Cys Arg Pro Lys Lys Asp Arg Ala Arg  
 Gln Glu Lys Lys Ser Val Arg Gly Lys Gly Lys Gly Gln Lys  
 20 Arg Lys Arg Lys Lys Ser Arg Tyr Lys Ser Trp Ser Val Pro  
 Cys Gly Pro Cys Ser Glu Arg Arg Lys His Leu Phe Val Gln  
 Asp Pro Gln Thr Cys Lys Cys Ser Cys Lys Asn Thr Asp Ser  
 25 Arg Cys Lys Ala Arg Gln Leu Glu Leu Asn Glu Arg Thr Cys  
 Arg Cys Asp Lys Pro Arg Arg.

30

2. Facteur de perméabilité vasculaire humain selon la revendication 1, qui est N-glycosylé au niveau du résidu d'acide aminé Asn 75.
3. Facteur de perméabilité vasculaire humain selon la revendication 1, qui est non glycosylé.
- 35 4. Facteur de perméabilité vasculaire humain selon la revendication 1, comprenant une séquence signal à l'extrémité N-terminale comme suit :

40 <sup>-26</sup> Met Asn Phe Leu Leu Ser <sup>-20</sup> Trp Val His Trp Ser Leu  
<sup>-10</sup> Ala Leu Leu Leu Tyr Leu His His Ala Lys Trp Ser Gln Ala.

45

5. ADNc du facteur de perméabilité vasculaire humain comprenant une séquence nucléotidique se présentant essentiellement comme suit :

151           ATG AAC TTT CTG CTG TCT TGG GTG CAT TGG AGC CTT  
 193   GCC TTG CTG CTC TAC CTC CAC CAT GCC AAG TGG TCC CAG GCT  
 235   GCA CCC ATG GCA GAA GGA GGA GGG CAG AAT CAT CAC GAA GTG  
 277   GTG AAG TTC ATG GAT GTC TAT CAG CGC AGC TAC TGC CAT CCA  
 319   ATC GAG ACC CTG GTG GAC ATC TTC CAG GAG TAC CCT GAT GAG  
 361   ATC GAG TAC ATC TTC AAG CCA TCC TGT GTG CCC CTG ATG CGA  
 403   TGC GGG GGC TGC TGC AAT GAC GAG GGC CTG GAG TGT GTG CCC  
 445   ACT GAG GAG TCC AAC ATC ACC ATG CAG ATT ATG CGG ATC AAA  
 487   CCT CAC CAA GGC CAG CAC ATA GGA GAG ATG AGC TTC CTA CAG  
 529   CAC AAC AAA TGT GAA TGC AGA CCA AAG AAA GAT AGA GCA AGA  
 571   CAA GAA AAA AAA TCA GTT CGA GGA AAG GGA AAG GGG CAA AAA  
 613   CGA AAG CGC AAG AAA TCC CGG TAT AAG TCC TGG AGC GTT CCC  
 655   TGT GGG CCT TGC TCA GAG CGG AGA AAG CAT TTG TTT GTA CAA  
 697   GAT CCG CAG ACG TGT AAA TGT TCC TGC AAA AAC ACA GAC TCG  
 739   CGT TGC AAG GCG AGG CAG CTT GAG TTA AAC GAA CGT ACT TGC  
 781   AGA TGT GAC AAG CCG AGG CGG

- 35
6. Plasmide VPfH/pUC9 tel qu'indiqué à la Figure 4, comprenant l'ADNc du facteur de perméabilité vasculaire humain tel que défini à la revendication 5.
  7. Clone contenant l'ADNc du facteur de perméabilité vasculaire humain de 3.5 kb environ comprenant l'ADNc selon la revendication 5.
  8. Cellules mammifères transfectées par un vecteur contenant la séquence d'ADNc du facteur de perméabilité vasculaire humain selon la revendication 5.
  9. Cellules C-127 murines transfectées par un vecteur contenant la séquence d'ADNc du facteur de perméabilité vasculaire humain selon la revendication 5.
  11. Procédé pour la production du facteur de perméabilité vasculaire humain *in vitro* comprenant la préincubation des cellules dérivées de la lignée cellulaire de lymphome histiocytique humain U-937 dans du milieu de culture nutritif à environ 35°C à 38°C pendant suffisamment longtemps pour permettre l'élaboration de facteur de perméabilité vasculaire et l'isolement du facteur de perméabilité vasculaire produit à partir des cellules usées ou du milieu de culture, le facteur de perméabilité vasculaire obtenu étant purifié par chromatographie échangeuse de cations, la purification étant effectuée à l'aide d'un tampon à pH 4,5, et le facteur de perméabilité vasculaire purifié étant lyophilisé.

(a) de chromatographie échangeuse de cations dudit milieu de culture cellulaire conditionné.

- (b) de chromatographie d'affinité sur support métallique des fractions douées d'activité VPF obtenues par ladite chromatographie échangeuse de cations; et  
(c) de CLHP en phase inverse des fractions douées d'activité VPF obtenues par ladite chromatographie d'affinité sur support métallique.

5

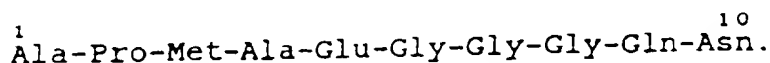
12. Procédé selon la revendication 11, dans lequel le milieu de culture nutritif est exempt de sérum.

10

13. Procédé selon la revendication 11, dans lequel la chromatographie échangeuse de cations est réalisée sur colonne de Fast Flow S-Sepharose échangeuse de cations, la chromatographie d'affinité sur support métallique est réalisée sur colonne cuivre/acide immunodiacétique (IDA)/Sepharose, et la CLHP en phase inverse est réalisée sur colonne CLHP en phase inverse en C<sub>18</sub>.

15

14. Facteur de perméabilité vasculaire humain ayant un poids moléculaire de 34 à 42 kDa environ tel que déterminé par SDS - PAGE en milieu non réducteur et une séquence d'acides aminés N-terminale comme suit :



20

15. VPF humain selon la revendication 14 contenant les séquences internes suivantes :

25

- (a) (Gln)Gln Gln Lys Pro,
- (b) (Arg)Gln Glu Gln Arg(Pro Lys),
- (c) Phe Met Asp Val Tyr Gln Arg(Arg),
- (d) Ile Phe Lys Pro Ser Cys Val Pro Leu Met Arg, and
- (e) Val Asp Ile Phe Gln Glu Tyr Pro Asp Glu Ile Glu Tyr.

#### Revendications pour l'Etat contractant suivant : ES

30

1. Procédé qui comprend la production de facteur de perméabilité vasculaire humain dont la séquence en acides aminés est la suivante :

35

40

45

Ala Pro Met Ala Glu Gly Gly Gly Gln Asn His His Glu Val  
 Val Lys Phe Met Asp Val Tyr Gln Arg Ser Tyr Cys His Pro  
 5 Ile Glu Thr Leu Val Asp Ile Phe Gln Glu Tyr Pro Asp Glu  
 Ile Glu Tyr Ile Phe Lys Pro Ser Cys Val Pro Leu Met Arg  
 10 Cys Gly Gly Cys Cys Asn Asp Glu Gly Leu Glu Cys Val Pro  
 Thr Glu Glu Ser Asn Ile Thr Met Gln Ile Met Arg Ile Lys  
 Pro His Gln Gly Gln His Ile Gly Glu Met Ser Phe Leu Gln  
 15 His Asn Lys Cys Glu Cys Arg Pro Lys Lys Asp Arg Ala Arg  
 Gln Glu Lys Lys Ser Val Arg Gly Lys Gly Lys Gly Gln Lys  
 20 Arg Lys Arg Lys Lys Ser Arg Tyr Lys Ser Trp Ser Val Pro  
 Cys Gly Pro Cys Ser Glu Arg Arg Lys His Leu Phe Val Gln  
 25 Asp Pro Gln Thr Cys Lys Cys Ser Cys Lys Asn Thr Asp Ser  
 Arg Cys Lys Ala Arg Gln Leu Glu Leu Asn Glu Arg Thr Cys  
 Arg Cys Asp Lys Pro Arg Arg.

- 30
2. Procédé selon la revendication 1, dans lequel le facteur de perméabilité vasculaire humain est N-glycosylé au niveau du résidu d'acide aminé Asn 75.
  - 35 3. Procédé selon la revendication 1, dans lequel le facteur de perméabilité vasculaire humain est non glycosylé.
  4. Procédé selon la revendication 1, dans lequel le facteur de perméabilité vasculaire humain comprend une séquence signal à l'extrémité N-terminale comme suit :

40                   <sup>-26</sup>                                   <sup>-20</sup>  
                   Met Asn Phe Leu Leu Ser Trp Val His Trp Ser Leu

<sup>-10</sup>  
                   Ala Leu Leu Leu Tyr Leu His His Ala Lys Trp Ser Gln Ala.

- 45
5. Procédé qui comprend la production d'ADNc du facteur de perméabilité vasculaire humain comprenant une sé-

151           ATG AAC TTT CTG CTG TCT TGG GTG CAT TGG AGC CTT  
 193   GCC TTG CTG CTC TAC CTC CAC CAT GCC AAG TGG TCC CAG GCT  
 5   235   GCA CCC ATG GCA GAA GGA GGA GGG CAG AAT CAT CAC GAA GTG  
 277   GTG AAG TTC ATG GAT GTC TAT CAG CGC AGC TAC TGC CAT CCA  
 10   319   ATC GAG ACC CTG GTG GAC ATC TTC CAG GAG TAC CCT GAT GAG  
 361   ATC GAG TAC ATC TTC AAG CCA TCC TGT GTG CCC CTG ATG CGA  
 403   TGC GGG GGC TGC TGC AAT GAC GAG GGC CTG GAG TGT GTG CCC  
 15   445   ACT GAG GAG TCC AAC ATC ACC ATG CAG ATT ATG CGG ATC AAA  
 487   CCT CAC CAA GGC CAG CAC ATA GGA GAG ATG AGC TTC CTA CAG  
 20   529   CAC AAC AAA TGT GAA TGC AGA CCA AAG AAA GAT AGA GCA AGA  
 571   CAA GAA AAA AAA TCA GTT CGA GGA AAG GGA AAG GGG CAA AAA  
 613   CGA AAG CGC AAG AAA TCC CGG TAT AAG TCC TGG AGC GTT CCC  
 25  
 655   TGT GGG CCT TGC TCA GAG CGG AGA AAG CAT TTG TTT GTA CAA  
 697   GAT CCG CAG ACG TGT AAA TGT TCC TGC AAA AAC ACA GAC TCG  
 30   739   CGT TGC AAG CCG AGG CAG CTT GAG TTA AAC GAA CGT ACT TGC  
 781   AGA TGT GAC AAG CCG AGG CGG  
 35

6. Procédé qui comprend la production du plasmide VPFh/pUC9 tel qu'indiqué à la Figure 4, comprenant l'ADNc du facteur de perméabilité vasculaire humain tel que défini à la revendication 5.
7. Procédé qui comprend la production d'un clone contenant l'ADNc du facteur de perméabilité vasculaire humain de 3.5 kb environ comprenant l'ADNc selon la revendication 5.
8. Cellules mammifères transfectées par un vecteur contenant la séquence d'ADNc du facteur de perméabilité vasculaire humain selon la revendication 5.
9. Cellules C-127 murines transfectées par un vecteur contenant la séquence d'ADNc du facteur de perméabilité vasculaire humain selon la revendication 5.

11. Procédé pour la production du facteur de perméabilité vasculaire humain in vitro comprenant la prolifération des cellules dérivées de la lignée cellulaire de lymphome histiocytique humain U-937 dans du milieu de culture nutritif à 37°C à 38°C pendant suffisamment longtemps pour permettre l'élaboration de facteur de perméabilité vasculaire humain, le facteur de perméabilité vasculaire produit étant des peptides vasoactifs, le milieu de culture étant conditionné dans lequel le facteur de perméabilité vasculaire est soluble et le milieu de culture cellulaire conditionné au moyen

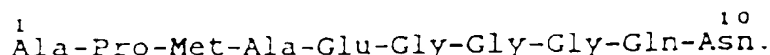


- (a) de chromatographie échangeuse de cations dudit milieu de culture cellulaire conditionné;  
 (b) de chromatographie d'affinité sur support métallique des fractions douées d'activité VPF obtenues par ladite chromatographie échangeuse de cations; et  
 (c) de CLHP en phase inverse des fractions douées d'activité VPF obtenues par ladite chromatographie d'affinité sur support métallique.

12. Procédé selon la revendication 11, dans lequel le milieu de culture nutritif est exempt de sérum.

13. Procédé selon la revendication 11, dans lequel la chromatographie échangeuse de cations est réalisée sur colonne de Fast Flow S-Sepharose échangeuse de cations, la chromatographie d'affinité sur support métallique est réalisée sur colonne cuivre/acide immunodiacétique (IDA)/Sepharose, et la CLHP en phase inverse est réalisée sur colonne CLHP en phase inverse en C<sub>18</sub>.

14. Procédé qui comprend la production d'un facteur de perméabilité vasculaire humain ayant un poids moléculaire de 34 à 42 kDa environ tel que déterminé par SDS - PAGE en milieu non réducteur et une séquence d'acides aminés N-terminale comme suit :



15. Procédé selon la revendication 14, dans lequel le VPF humain contient les séquences internes suivantes :

- (a) (Gln)Gln Gln Lys Pro,  
 (b) (Arg)Gln Glu Gln Arg(Pro Lys),  
 (c) Phe Met Asp Val Tyr Gln Arg(Arg),  
 (d) Ile Phe Lys Pro Ser Cys Val Pro Leu Met Arg, and  
 (e) Val Asp Ile Phe Gln Glu Tyr Pro Asp Glu Ile Glu Tyr.

## HUMAN VPF PURIFICATION SCHEME

U937 SERUM FREE CONDITIONED MEDIUM



CONCENTRATE 6X (AMICON)



S-SEPHAROSE FAST FLOW  
CATION EXCHANGE CHROMATOGRAPHY  
(NaCl GRADIENT ELUTION)



CONCENTRATE 20X (AMICON)



SEPHAROSE FAST FLOW  
IDA  $\text{Cu}^{++}$  CHELATE CHROMATOGRAPHY  
(IMIDAZOLE GRADIENT ELUTION)



$\text{C}_{18}$  RPHPLC  
( $\text{H}_2\text{O}$ /TFA/ $\text{CH}_3\text{CN}$  GRADIENT ELUTION)

YIELD: 1-5 µg/4L CONDITIONED MEDIUM  
PURIFICATION: 10,000-100,000 FOLD

**FIG.1.**

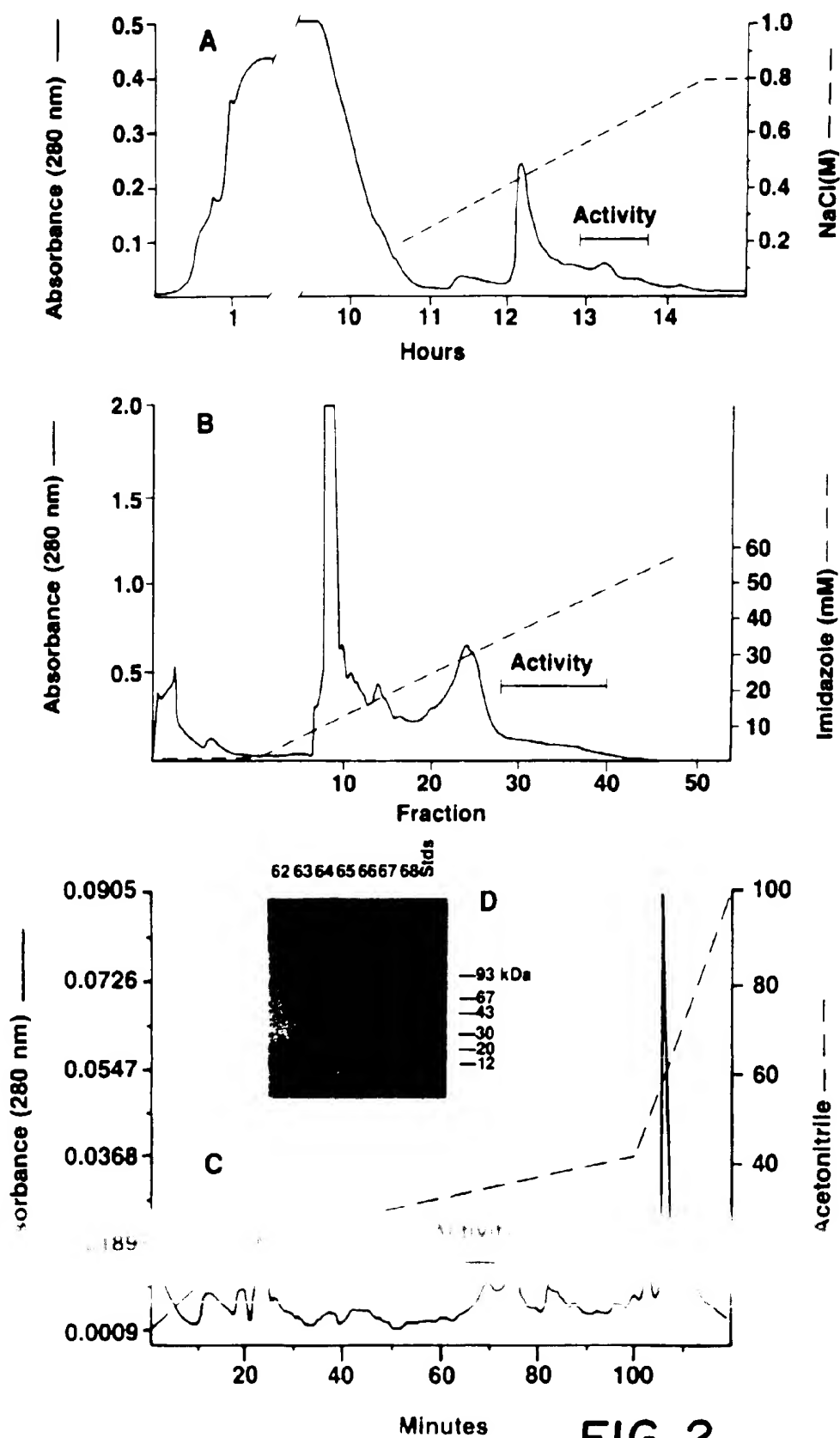


FIG. 2

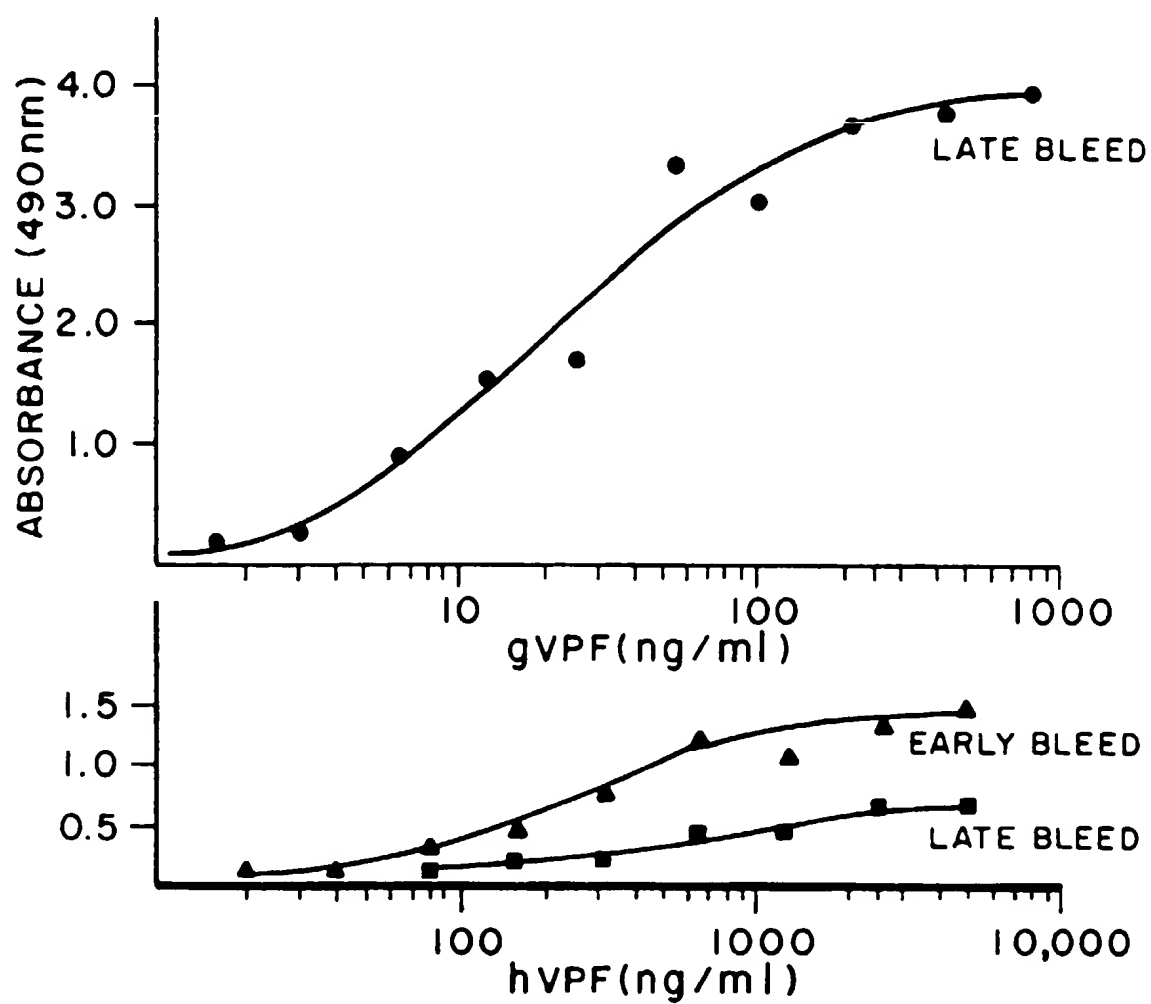


FIG. 3.

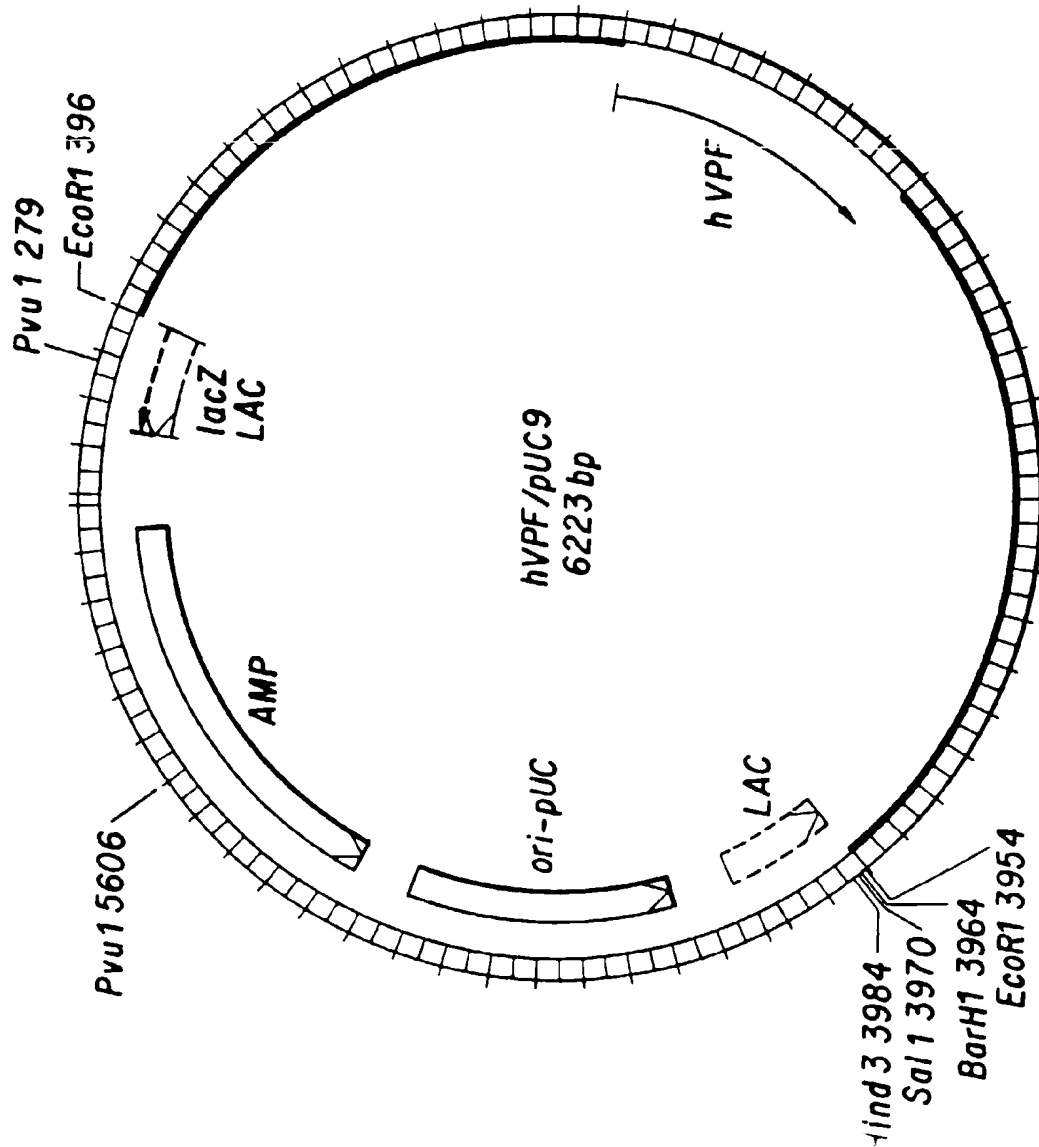


FIG. 4